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The Chromophore and Polypeptide Composition of *Aplysia* Ink

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Abstract. The composition of the ink of the sea hare, *Aplysia*, was studied in regard to its tetrapyrrole and polypeptide content. The ink was separated into three pigment components by both thin-layer and gel filtration chromatography. These three pigments have distinctive visible absorption spectra, and—by comparison with known tetrapyrroles—we have demonstrated that they are derived from the three tetrapyrrole chromophores (bilins) found on the biliproteins of certain red algae, which constitute a portion of the *Aplysia* diet. The red component is phycourobilin; the purple is phycoerythrobilin; and the blue is phycocyanobilin. Sodium dodecyl sulfate gel electrophoresis experiments were also performed. The results of these experiments showed several polypeptides, and major bands at 78,000 and 61,000 molecular weight were noted. Biliproteins, at most, would be minor components of the ink.

Introduction

Aplysia, the sea hare, is a marine mollusk whose principal habitats are the littoral and sublittoral regions, where it feeds on various seaweeds [see Kandel (1979) for a review]. *Aplysia* discharge a dark purple ink from a gland (called the ink, purple, or Blochmann's gland) located on the edge of its mantle shelf. The ink has been viewed by some biologists as a defense against predators, but there is no consensus on how it would perform this function. Eales (1921) suggested that the ink is a screen used by the *Aplysia* to cloud its escape from danger. Others have disagreed, noting that an ink cloud might be effective in shallow, calm waters but not in sublittoral water, where it would be diluted too quickly to protect the slow-moving sea hare (Carew and Kandel, 1977). Others concluded that the ink would not be effective as a screen even in shallow water (Kupfermann and Carew, 1974; Linton, 1966). DiMatteo (1981, 1982) found that the ink alone, or when injected into food, is avoided by seagulls and crabs, who apparently find it distasteful or toxic. Nondefense functions, such as excretion or signalling, have also been attributed to the ink (Chapman and Fox, 1969; Tobach *et al.*, 1965).

Unsuccessful attempts have been made to learn how the sea hare uses ink in its natural environment. Carew and Kupferman (1974) and Kupferman and Carew (1974) reported on lengthy observations of *Aplysia californica* in a variety of habitats; inking was never observed. Furthermore, they did not observe an *Aplysia* being attacked by a predator. Inking occurred routinely, however, if the mollusks were roughly handled by the investigators. These results suggest, but do not prove, that the ink is a defense mechanism used only in rather extreme cases. The function of the ink would then be produced by its chemical composition rather than its optical properties.

Inking has been studied by the techniques of neurophysiology (*e.g.*, Byrne, 1981), and it was determined that an electric-shock stimulus must cross a high threshold before inking occurs (Carew and Kandel, 1977).

Chapman and Fox (1969) studied the correspondence between diet and the presence of ink in *Aplysia*. After inducing complete discharge of the ink by tactile stimulation, they fed the spent organisms either brown or red algae and found that only after feeding with red algae was the ink replenished. Rüdiger (1967) had shown that the major pigment in the ink has the structure of a monomethyl ester of phycoerythrobilin, a chromophore of the

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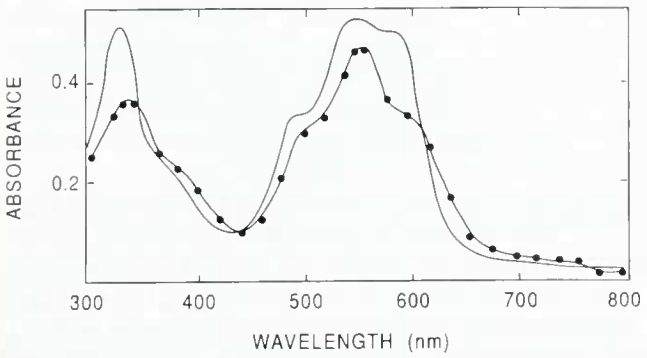

Figure 1. Absorption spectra of *Aplysia* inks. The visible spectra of inks from different animals may vary.

biliprotein phycoerythrin from red algae. Because brown algal seaweed are devoid of biliproteins, it is probable that the ink pigments are derived from the biliproteins of the red algae. Because these chromophores are covalently attached to protein via one or two thioether linkages to cysteine (for references, see MacColl and Guard-Friar, 1987), *Aplysia* must therefore cleave the chromophores off the biliproteins and store the pigments in the ink gland. Troxler *et al.* (1981) obtained some ink pigments that were still attached to a cysteine residue. The main pigment component was 90% free and 10% cysteine-bound. We have now investigated the identities of the other chromophores in the ink.

Materials and Methods

Aplysia californica was obtained from Marine Specimens Unlimited (Pacific Palisades, California) and were inked immediately upon arrival either by dissection or by physical stimulation. Care was taken to obtain ink that was free of secretion from the opaline gland. The thick, white, mucus-like substance from the opaline gland was easily observed if secreted, but usually in our protocols the ink gland secreted with no secretion of the opaline. Any ink contaminated by this substance was discarded. The ink was stored frozen until needed for our experiments.

The chromophore components of the ink were separated by two methods, thin layer chromatography and gel filtration chromatography. Thin-layer chromatography of the ink was performed on 5 × 10 cm Silica gel 60 F254 precoated plates. The chromatograms were developed with a solution of 50% benzene: 35% methanol: 15% ethyl acetate. The gel filtration experiments were carried out using Ultrogel AcA54 (LKB) with pH 6.0, 0.1 ionic strength, sodium phosphate buffer with 0.5 *M* NaCl added. Some plates were likewise developed in a second direction.

B-Phycoerythrin was isolated from the red alga, *Porphyridium cruentum*. The alga was grown in our laboratory, harvested, and stored frozen. Cells were broken in a French pressure cell, and the water-soluble proteins extracted into pH 6.0, 0.1 ionic strength, sodium phosphate buffer. The B-phycoerythrin was purified by ammonium sulfate fractionation (50% saturated ammonium sulfate), gel-filtration column chromatography on Sepharose 6B (Pharmacia), and chromatography on a hydroxylapatite column (Bio-gel HT, Bio-Rad). The ratios of the absorbance of B-phycoerythrin at its visible maximum compared with both the visible absorbance maxima of the other biliproteins and 280 nm were used to determine

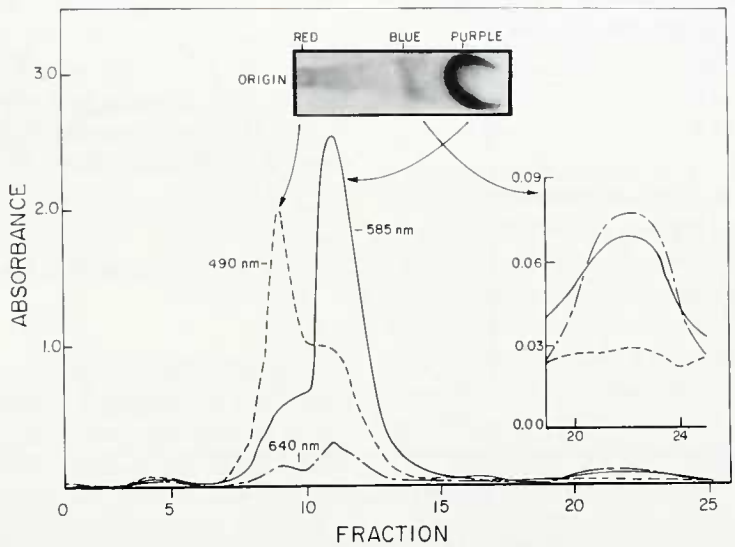

Figure 2. Chromatographic separation of *Aplysia* ink into three pigments. The TLC is shown as a photograph, and the Ultrogel elution pattern is plotted as absorbance *versus* fraction number. The inset shows the absorbances of these fractions on an expanded scale. Pools of ink from several sea hares were used in these experiments.

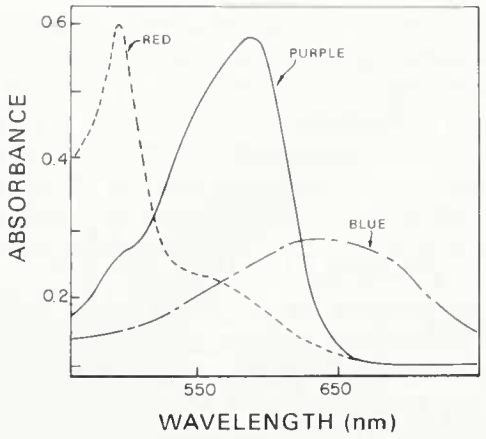

Figure 3. Visible absorption spectra of the three ink pigments. The solvent is sodium phosphate buffer, pH 6.0, with 0.50 *M* sodium chloride.

purity. The hydroxylapatite chromatography was performed as described previously (MacColl *et al.*, 1981), and the B-phycoerythrin eluted prior to the other biliproteins. Purified protein was dialyzed into distilled water, lyophilized, and stored in a refrigerator.

B-Phycoerythrin is composed of three subunits, α and β (17,500 molecular weight) and γ (30,000 molecular weight). The subunits were dissociated in 8.0 *M* urea, pH 3.0 and separated on a Sephacryl S-200 (Pharmacia) column in the same solvent. Fractions containing γ subunit were identified spectroscopically on the basis of its characteristic phycourobilin absorbance at 490 nm. The γ subunit has both phycourobilin and phycoerythrobilin chromophores while the α and β subunit have only phycoerythrobilins (see MacColl and Guard-Friar, 1987, for references). The fractions containing γ subunit were then rechromatographed on the same column to complete their purification.

C-Phycocyanin was isolated from the blue-green alga, *Phormidium luridum*, after treating the cells with the enzyme lysozyme. The protein was purified by ammonium sulfate precipitation, first with 50% followed by 35% saturated ammonium sulfate. Purified protein was dialyzed into distilled water, lyophilized, and stored in a refrigerator. C-Phycocyanin, which has only phycocyanobilin for chromophores, was refluxed in methanol overnight. The reflux mixture was filtered and the blue solution, containing phycocyanobilin, was evaporated to dryness. The absorption spectrum of the protein-free phycocyanobilin was obtained in acidic methanol. Absorption spectra were obtained at room temperature using a model 320 spectrophotometer (Perkin-Elmer).

Sodium dodecyl sulfate gel electrophoresis was performed using precast, 10–20% polyacryamide gradient gels (Geltech, Salem, Ohio). The ink was dialyzed into distilled water and lyophilized. The lyophilized material was suspended in pH 6.0, 0.1 ionic strength, sodium phosphate buffer with 1% sodium dodecyl sulfate. The reconstituted ink was treated with a sample buffer containing: 50 parts distilled water; 12.5 parts 0.3 *M* Tris HCl, pH 6.8; 10 parts glycerol; 20 parts (10% w/v) sodium dodecyl sulfate; 5 parts β-mercaptoethanol; and 2.5 parts (0.05% w/v)

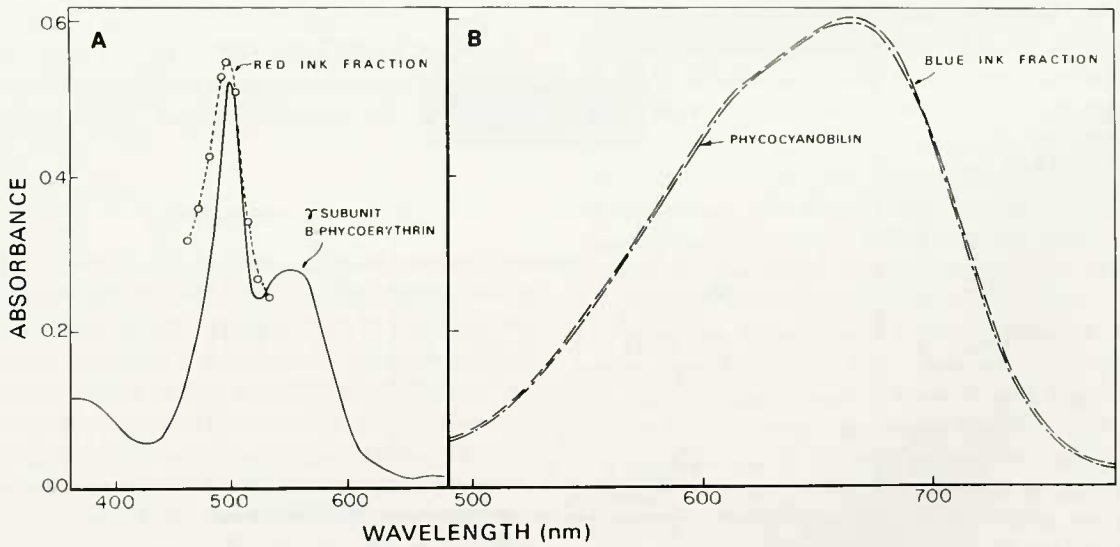

Figure 4. Spectroscopic comparisons of *Aplysia* pigments with biliprotein chromophores. (A) Comparison of red pigment with γ subunit of B-phycoerythrin. Phycourobilin has its absorption maximum near 490 nm, and phycoerythrobilin is near 560 nm. The solvent is 8.0 *M* urea, pH 3.0. This solvent minimizes the effect of the apoprotein on the spectrum of the bilin. (B) Comparison of blue pigment with phycocyanobilin cleaved from C-phycocyanin both in acidic methanol.

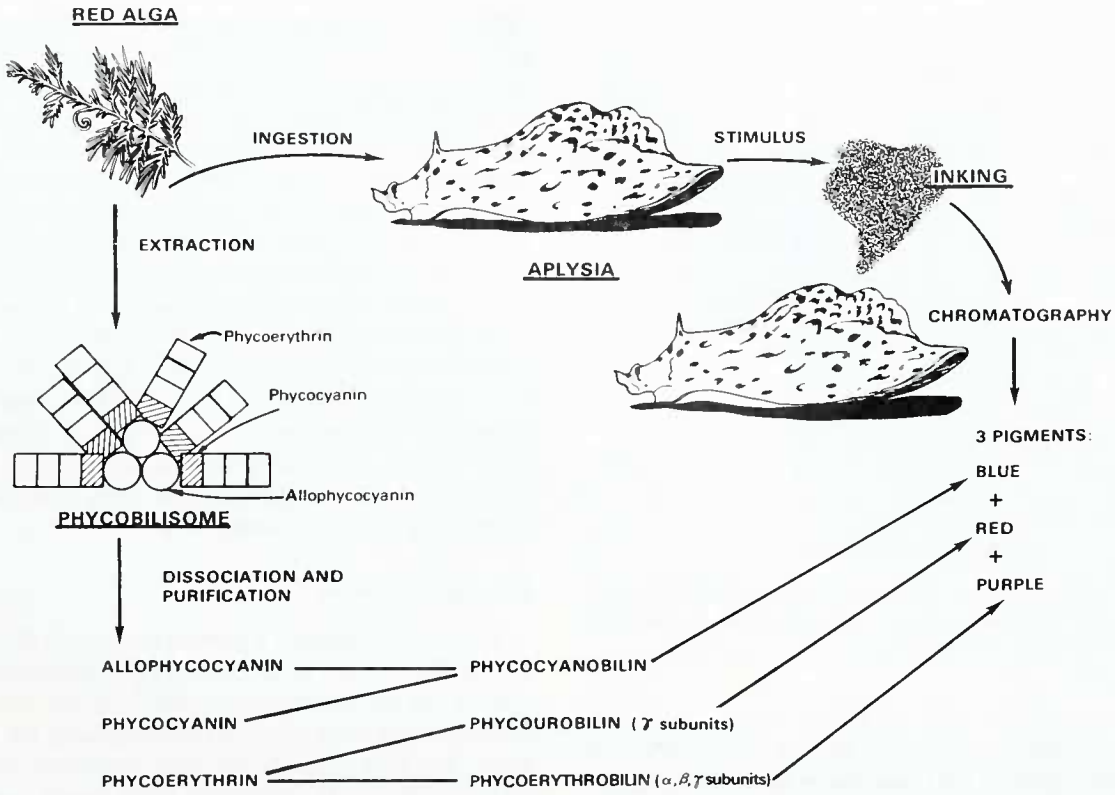

Figure 5. Schematic representation of derivation of pigments of *Aplysia* ink from phycobilisomes of a hypothetical red alga. Although not indicated on the diagram, B-phycoerythrin has phycourobilin only on the γ subunit, while R-phycoerythrin has phycourobilin on both the γ and β subunits. C-Phycocyanin has only phycocyanobilins, while R-phycocyanin has both phycocyanobilin and phycoerythrobilin. (For references, see MacColl and Guard-Friar, 1987.)

Bromophenol Blue. The final concentration of ink was 100 μg in the 20 μl of heated sample which was applied to each lane. The gels were stained with Coomassie blue, and destained with 50% distilled water; 40% methanol; 10% acetic acid. The gels were then stored in a 5% glycerol solution. Standards used for molecular weight calibration were: bovine serum albumin, ovalbumin, chymotrypsinogen, and lysozyme.

Results and Discussion

Chromophore content

Visible absorption spectra of *Aplysia* inks (Fig. 1) show a number of bands. The relative intensities of the various band maxima vary with ink source; two typical results are shown (Fig. 1). Earlier studies on the chromophore content of the ink have yielded variable results (Christomanos, 1955; Winkler, 1959; Chapman *et al.*, 1967; Lederer and Huttrer, 1942; Schreiber, 1929; Nishibori, 1960). We have, therefore, chosen to separate the ink chromophores by two entirely distinct methods: thin-layer (nonaqueous) and gel filtration (aqueous, pH 6.0) chromatography. Thin-layer chromatography (TLC) separated the ink into three colored components. The fastest-migrating ($R_f = 0.53$) was purple; the middle component ($R_f = 0.40$) was blue; and the third, which remained near the origin ($R_f = 0.07$), was red. When a TLC plate was run in two dimensions, a yellow component was also observed in the vicinity of the blue. Gel filtration produced very similar results; the brownish-red material eluted first, followed by purple, and finally by a light blue band (Fig. 2). The major purple and red fractions were rechromatographed on the same Ultrogel column, and the absorption spectra of the three purified components were obtained (Fig. 3). The red pigment showed a prominent absorption band at 490 nm, the purple maximum was at 585 nm, and the blue at 640 nm.

The absorption spectrum of the brownish-fed component, which was found by both thin-layer and Ultrogel chromatography, was compared with the absorption spectrum of a biliprotein chromophore—phycourobilin—obtained from β-phycoerythrin (Fig. 4A). Phycourobilin is covalently attached to the γ subunits of B-phycoerythrin, which was purified by chromatography on Sephacryl

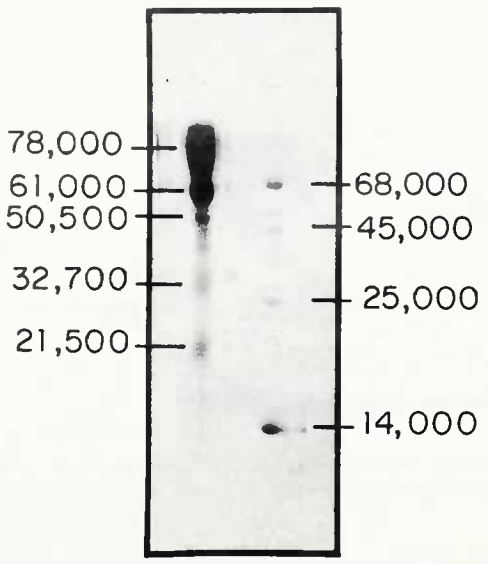

Figure 6. Sodium dodecyl sulfate gel electrophoresis results of *Aplysia* ink. On the right are molecular weight standards, and on the left are the polypeptides from a pool of ink.

S-200 in the presence of 8.0 *M* urea at pH 3.0. Phycourobilin has its absorption maximum near 490 nm, and its absorption spectrum shows also that phycoerythrobilin also occurs on this subunit. Considering these two differences—that phycourobilin is covalently attached to a polypeptide and that it occurs together with phycoerythrobilin on that subunit—the agreement between the absorption spectra of phycourobilin and the red ink component is satisfactory. The acidic urea solvent minimizes the effects of apoprotein on the spectra of the bilins.

The blue component of the ink was also purified by column chromatography. Its spectrum is identical to that of authentic phycocyanobilin (Fig. 4B), which was obtained by methanol refluxing of C-phycocyanin. The structure of phycocyanobilin has been determined by Cole *et al.* (1967). The spectrum of phycocyanobilin is highly dependent on solvent, and near neutral pH the absorption maximum is blue shifted to around 600 nm. The pH of the ink is about 6.54 ± 0.35.

The three pigments of *Aplysia* ink therefore appear to be derived, apparently without extensive chemical modification, from three algal biliprotein chromophores. The precise diet of the *Aplysia* used in our studies is unknown, but we can assume that a representative red alga is a component of the diet. In this seaweed a typical selection of biliproteins—B-phycoerythrin (or R-phycoerythrin), C-phycocyanin (or R-phycocyanin), and allophycocyanin—would be arranged in phycobilisomes (Fig. 5). The C-phycocyanin and allophycocyanin have as their chromophore phycocyanobilin, which becomes the blue ink component. B-Phycoerythrins (or R-phycoerythrins) have both phycoerythrobilin and phycourobilin, which become the purple and red ink components, respectively. Several phycoerythrobilins per phycourobilin can be found on each phycoerythrin aggregate (for B-phycoerythrin the ratio is 3.5 to 1). Most red algal phycobilisomes have more phycoerythrin than the sum of phycocyanin plus allophycocyanin (Fig. 5), and each phycoerythrin aggregate has more chromophores than do similarly sized aggregates of allophycocyanin or C-phycocyanin. The predominance of the purple component over the blue is thus a function of the structure of the red algal phycobilisome. In addition, Chapman and Fox (1969) found some phycocyanobilin to be selectively localized in the skin of the *Aplysia*. Variability in the color of the ink (*e.g.*, Winkler, 1959) is most likely related to the feeding of individuals on different types of red algae since the various algae show biliprotein diversity in their phycobilisomes.

Polypeptide content

Studies examining the protein content of the ink were conducted. Sodium dodecyl sulfate gel electrophoresis was carried out on proteins extracted from the ink (Fig. 6). Several bands were observed after staining for polypeptides. The two main bands have molecular weights of 78,000 and 61,000. The other three minor bands have molecular weight values of 50,500, 32,700, and 21,500. Biliproteins have polypeptides ranging from 15,000 to 22,000 (MacColl and Guard-Friar, 1987), and they are apparently not major ink constituents. Additional immunochemical experiments are needed to discover whether any biliproteins are present in the ink.

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